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Rapid Method for Separation of Bacterial DNA from Humic Substances in Sediments for Polymerase Chain Reaction

YU-LI TSAI[1]* AND BETTY H. OLSON[2]

County Sanitation Districts of Orange County, Fountain Valley, California 92728,[1] and Program in Social Ecology, University of California, Irvine, California 92717[2]

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The polymerase chain reaction (PCR) was used to amplify an *Escherichia coli* 16S ribosomal gene fragment from sediments with high contents of humic substances. Total DNA was extracted from 1 g of *E. coli* seeded or unseeded samples by a rapid freeze-and-thaw method. Several approaches (use of Bio-Gel P-6 and P-30 and Sephadex G-50 and G-200 columns, as well as use of the Stoffel fragment) were used to reduce interference with the PCR. The best results were obtained when crude DNA extracts containing humic substances were purified by using Sephadex G-200 spun columns saturated with Tris-EDTA buffer (pH 8.0). Eluted fractions were collected for PCR analyses. The amplified DNA fragment was obtained from seeded sediments containing fewer than 70 *E. coli* cells per g. Because only 1/100 of the eluted fractions containing DNA extracts from 70 cells per g was used for the PCR, the sensitivity of detection was determined to be less than 1 *E. coli* cell. Thus, DNA direct extraction coupled with this technique to remove interference by humic substances and followed by the PCR can be a powerful tool to detect low numbers of bacterial cells in environmental samples containing humic substances.

The polymerase chain reaction (PCR) is a very powerful and sensitive analytical technique with applications in many diverse fields, including molecular biology (11, 14, 22), clinical diagnosis (1, 15, 16, 23), forensic analysis (8, 9), and population genetics (3). Recently, the PCR has been applied to the field of environmental sciences to detect coliforms, fecal coliforms, and pathogenic microorganisms in water samples (4–6, 12, 13).

Concentrating large volumes of water is an essential step when screening water samples for pathogens by using the PCR. Because of their chemical characteristics, humic substances are also concentrated during this process and subsequently interfere with the PCR. Humic substances, mainly humic acid and fulvic acid, are commonly found in aquatic, soil, and sediment environments (18). It has been reported previously that trace amounts of humic substances can inhibit the PCR and cause false-negative results (21). In addition, it was also found that humic and fulvic acids reduced the efficiency of adsorption and the recovery of poliovirus from water (17). Since humic substances become a major concern upon the amplification of target DNA extracted from soil or sediment samples, it is important that humic substances be removed or attenuated from the nucleic acid extracts to avoid inhibition of the PCR. In a previous report, we used diluted humic extracts as templates in the PCR to successfully detect the presence of low numbers of bacterial cells in soil and sediments (21). However, by this approach the sensitivity of detection decreased because of the dilution of template to reduce interference by humic substances. In the present work, we applied a rapid gel filtration method to separate DNA from extracts containing humic substances, which resulted in good PCR amplification. Because the samples were not diluted before the PCR, an increase in sensitivity compared with that of the previous method was observed.

* Corresponding author.

MATERIALS AND METHODS

Sampling. Sediments were collected from a settling pond in Oak Ridge, Tenn. (ORT), and were stored at 4°C before analysis. These samples (pH 8.0) were, on average, 51% water and possessed high cation-exchange capacity (34 meq/100 g) and low concentrations of sand. The weights of all test samples were expressed as wet weights throughout the experiments. Total DNA was extracted from 1 g (0.49 g [dry weight]) of sediments by using a rapid method (19) and was resuspended in 200 μl of sterile Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0). The humic extracts were obtained from sterile unseeded sediments by using the same DNA direct extraction method. The humic-acid-like substances were obtained by using centrifugation, and the pellets were air dried before quantitation. Commercially available humic acid (Aldrich Chemical Co., Milwaukee, Wis.) was used as a standard control.

Organism. *Escherichia coli* 35346 (ECOR27) was obtained from the American Type Culture Collection (Rockville, Md.) and maintained on nutrient agar (Difco, Detroit, Mich.). ECOR27 cells at late exponential phase were seeded into sterile sediment samples and enumerated by using heterotroph plate count techniques (2).

PCR. Total DNA extracted from ECOR27 cells was employed as the template in the PCR. A "hot-start" PCR protocol, with the appropriate oligonucleotide primers for two 16S rRNA gene fragments (371 and 237 bp), was performed as described by Tsai and Olson (21). PCR products amplified from purified DNA were visualized as single bands on a 2% agarose gel stained with ethidium bromide. Additionally, in order to determine the impact of humic acid on the PCR, the magnesium ion-tolerant Stoffel fragment (Perkin-Elmer Cetus, Norwalk, Conn.), functioning as AmpliTaq polymerase but without exonuclease activity, was used with magnesium ion concentrations ranging from 2.5 to 11.25 mM. The thermocycling reaction parameters for the PCR were always 40 cycles; one cycle is expressed as 1.5 min at 95°C for denaturation and 1 min at 62°C for annealing and extension.

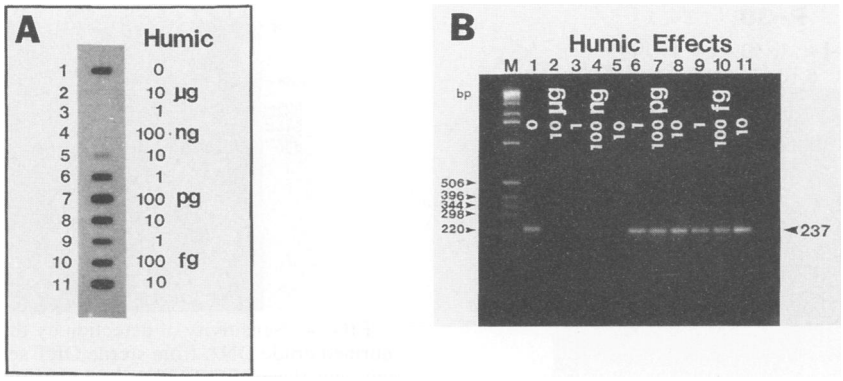

FIG. 1. Effects of humic acid on the PCR. Shown are an autoradiogram of amplified DNA hybridization against an internal oligonucleotide probe (A) and amplified 16S rRNA gene fragments (237 bp) on an agarose gel stained with ethidium bromide (B). *E. coli* genomic DNA (30 ng) was used as a template in each reaction. The amounts of humic acid tested in the experiment are as indicated. Lane M, DNA 1-kb ladder marker (2 μg).

DNA hybridization. An internal oligonucleotide probe (20-mer) was used to verify the PCR product. The amplified DNA was transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) by slot blotting, and DNA hybridization was performed under high-stringency conditions as previously described (19, 21).

DNA purification by gel filtration. For DNA purification, crude DNA extracts from sediments were run through two types of spun columns. Aliquots (50 μl) were loaded onto TE buffer saturated Bio-Gel P-6 and P-30 (Bio-Rad Laboratories, Richmond, Calif.) polyacrylamide gel columns. Fifty microliters of eluents was collected after centrifugation at 1,100 × *g* for 10 min. Additionally, 5 ml of TE-saturated Sephadex G-50 and G-200 (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) was packed into 5-ml sterile syringes. To avoid false-positive results by the PCR, TE was autoclaved and filter sterilized and all glassware was autoclaved. A 10-min spin at 1,100 × *g* was carried out to remove excess TE buffer. The final columns contained 1.5 cm of gel beads with 1 cm of glass wool at the bottom. One hundred-microliter aliquots of crude extracts were slowly loaded onto the center of the column. One fraction (50 μl) of eluent was collected for each sample after the columns were centrifuged at 1,100 × *g* for 10 min, and another 10-min spin for one additional fraction (50 μl) followed. One microliter of pooled fraction (100 μl) was used as the PCR template to test for the presence of target DNA.

RESULTS AND DISCUSSION

Humic-acid-like substances were found in crude DNA extracts by using a freeze-and-thaw method (20). Because some humic-acid-like substances were lost during the phenol-chloroform extraction step, an average of 54 mg (dry weight) of humic-acid-like substances was determined in crude DNA extract from 1 g of ORT sediments. One microliter (27 μg of humic-acid-like substances) of undiluted extracts was sufficient to inhibit the PCR. For comparison, pure humic acid (Aldrich) can suppress the PCR at concentrations as low as 10 ng (Fig. 1). Thus, humic acid present in crude DNA extracts can cause major interference when performing the PCR. Other elements, such as metal ions (7) and fulvic acid, could also contribute to the inhibitory effects. Possible reasons for these problems were discussed in a previous note (21).

Figure 2 shows the amplified products after the crude extracts were purified with Bio-Gel P-6 and Sephadex G-50 columns. Undiluted DNA extracts (200 μl) were obtained from 1 g of sterile ORT sediment seeded with 1.5×10^8 ECOR27 cells. Because the recovery efficiency is greater than 90% (20), it is assumed that 1 μl of crude extract contains DNA amounting to 7.0×10^5 cells. No PCR products were observed by using eluents from either column as templates when reaction mixtures contained greater than 27 μg of humic-acid-like substances (Fig. 2, lanes 1 and 2 and 5 and 6). This indicates that neither column was able to remove humic-acid-like substances sufficiently to prevent inhibitory effects. However, positive amplification was found when the eluents were diluted 10- to 100-fold (Fig. 2, lanes 3 and 4 and 7 and 8). No amplification products were detected when target DNA sequences were not present in diluted eluents (Fig. 2, lane 9).

Figure 3 exhibits another example of the effects of humic

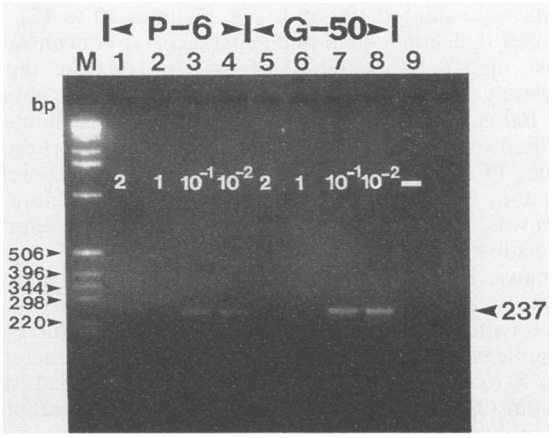

FIG. 2. Effects of humic substances extracted from sterile seeded sediments on the PCR. P-6 and G-50 represent eluents collected from a Bio-Gel P-6 column and a Sephadex G-50 column, respectively. Lanes (numbers in parentheses are amounts of humic-acid-like substances determined): M, DNA 1-kb ladder marker; 1 and 5, undiluted DNA extract (54 μg); 2 and 6, undiluted DNA extract (27 μg); 3 and 7, 10^{-1} diluted DNA extract (2.7 μg); 4 and 8, 10^{-2} diluted DNA extract (0.27 μg); 9, reaction mixture only, without template.

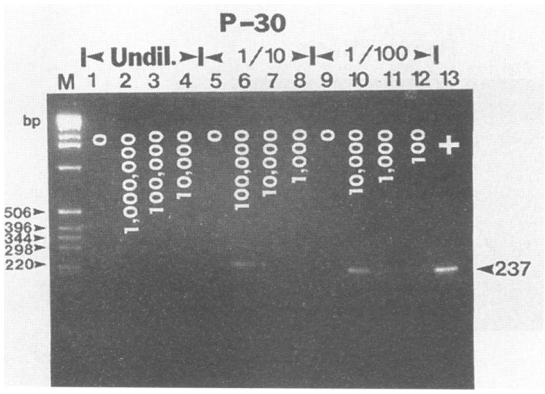

FIG. 3. Effects of Bio-Gel P-30-purified crude DNA extracts on PCR. Lanes (numbers in parentheses are bacterial cell counts for DNA extraction): M, DNA 1-kb ladder marker (2 μg); 1, undiluted crude extracts from sterile unseeded sediments; 2, undiluted crude extracts containing DNA extracted from 10^6 ECOR27 cells; 3, undiluted extracts (10^5 cells); 4, undiluted extracts (10^4 cells); 5, 10^{-1} diluted extracts from sterile unseeded sediments; 6, 10^{-1} diluted crude extracts containing DNA extracted from 10^5 ECOR27 cells; 7, 10^{-1} diluted extracts (10^4 cells); 8, 10^{-1} diluted extracts (10^3 cells); 9, 10^{-2} diluted extracts from sterile unseeded sediments; 10, 10^{-2} diluted crude extracts containing DNA extracted from 10^4 ECOR27 cells; 11, 10^{-2} diluted extracts (10^3 cells); 12, 10^{-2} diluted extracts (10^2 cells); 13, 30 ng of *E. coli* genomic DNA as the template.

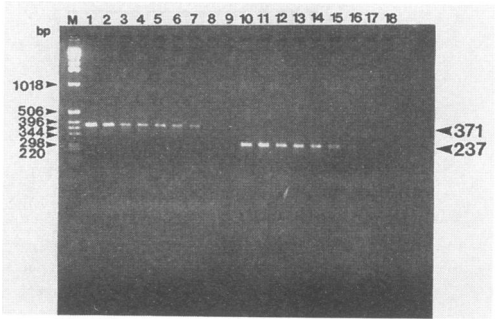

FIG. 4. Sensitivity of detection by the PCR on Sephadex G-200-purified crude DNA from sterile ORT sediments seeded with different densities of ECOR27 cells. Lanes (because only 1 μl of the 100 μl of pooled eluent was used for the PCR, the numbers shown in parentheses are the estimated counts of bacterial cells which contribute DNA for the PCR): M, DNA 1-kb ladder marker (1 μg); 1 and 10, 7.0×10^7 cells per g (7.0×10^5 cells); 2 and 11, 7.0×10^6 cells per g (7.0×10^4 cells); 3 and 12, 7.0×10^5 cells per g (7.0×10^3 cells); 4 and 13, 7.0×10^4 cells per g (7.0×10^2 cells); 5 and 14, 7.0×10^3 cells per g (70 cells); 6 and 15, 7.0×10^2 cells per g (7 cells); 7 and 16, 7.0×10^1 cells per g (0.7 cells); 8 and 17, 7 cells per g (0.07 cells); 9 and 18, sterile unseeded controls.

substances on the PCR by using eluent collected from Bio-Gel P-30 columns for amplification. The crude DNA extracts were from sterile ORT sediments seeded with ECOR27 cells at concentrations ranging from 2.0×10^8 to 2.0×10^{10} cells per g. One microliter of undiluted extract containing 27 μg of humic-acid-like substances and target DNA inhibited the PCR (Fig. 3, lanes 2 to 4). Amplified DNA fragments were detected when extracts diluted 10-fold (2.7 μg of humic-acid-like substances) containing bacterial DNA from 10^3 to 10^5 cells were used as templates (Fig. 3, lanes 6 to 8). Higher yields of PCR products were observed as the eluents were diluted 100-fold (Fig. 3, lanes 10 to 12). This indicated that humic-acid-like substances at concentrations of less than 0.27 μg/100 μl of reaction mixture did not completely block the PCR, and thus target DNA obtained from 100 cells was amplified. Negative controls (diluted or undiluted extracts from unseeded sterile sediment) did not produce PCR products (Fig. 3, lanes 1, 5, and 9). Therefore, when P-6, P-30, and G-50 columns were used, dilution of eluent was still necessary in order to attenuate the effects of humic substances on the PCR.

Because humic acids could chelate magnesium ions, required by *Taq* polymerase, and subsequently inhibit the PCR, an alternative AmpliTaq DNA polymerase, the Stoffel fragment, was tested. The Stoffel fragment is characterized by no 5′-to-3′ exonuclease activity (10) and a broad $MgCl_2$ optimum (2.5 to 5.0 mM) compared with that of AmpliTaq DNA polymerase. It generally requires higher concentrations of magnesium ions, as suggested by the manufacturer (Perkin-Elmer Cetus). It was evidenced that higher concentrations (5 to 10 mM) of Mg^{2+} cause some degree of inhibitory effects on AmpliTaq (data not shown). In a separate experiment, the effects of humic substances on the PCR with the Stoffel fragment in varied concentrations of magnesium ions were determined. *E. coli* genomic DNA (30 ng) served as a template in each reaction. After amplification, PCR products (237-bp fragments) were found when no humic substances were present in the reaction mixture with magnesium ion concentrations ranging from 2.5 to 11.25 mM. However, when the humic substances (humic acid, >10 ng; humic-acid-like substances, >2.7 μg) were present in the reaction mixture, no PCR product could be detected, even at high Mg^{2+} concentrations. No PCR product was found when the amplification was carried out with a small amount of humic acid (10 ng) and a high concentration of Mg^{2+} (11.25 mM). This illustrated that PCR inhibition probably was not due to chelation of humic acid or humic-acid-like substances with Mg^{2+}.

PCR results from the use of Sephadex G-200-purified crude DNA extracts (containing 27 μg of humic-acid-like substances per μl of crude extract) from sterile ORT sediments seeded with different cell densities (0 to 7.0×10^7 cells per g) are shown in Fig. 4. The results indicate that inhibitory effects were greatly reduced after purification with G-200 columns. The sensitivity of detection was determined to be 70 cells per g of sediments, as evidenced by both amplified fragments (Fig. 4). Since only 1 μl of the 100 μl of pooled eluent was used for amplification, the detection limit by the PCR can also be interpreted as less than 1 bacterial cell in template added initially for amplication (Fig. 4, lanes 7 and 16). Because one *E. coli* cell could contain seven copies of the 16S rRNA gene, it is possible that DNA target sequences from less than 1 cell could be amplified by the PCR. However, the above sensitivity measurements do not exclude the possible amplification of target DNA released from seeded dead *E. coli* cells. No amplification products werc found in sterile unseeded sediments or in sediments seeded with 7 cells per g. No PCR products were detected from sediments seeded with 7 cells per g, even when larger aliquots (5 to 10 μl) of eluent were used, indicating a possible lack of DNA templates in the eluent. Assuming the DNA extraction efficiency is 90% (20), 6.3 cells per g were extracted from sediment seeded with 7 cells per g. Positive PCR product was expected with a 10-μl aliquot as the template. However, the total DNA of 6.3 cells weighed approximately 57 fg, which could be reduced further during gel filtration and thus render sensitivity beyond detection by

the PCR. Positive results were obtained by using 10 μl of eluent from 70 cells per g of sediment as the template. These data show that Sephadex G-200 sufficiently separates humic-acid-like substances from crude DNA extracts so that larger amounts (10 μl) of eluent can be applied for the PCR without inhibitory effects.

In conclusion, Sephadex G-200 proved to be the best column tested for the separation of humic-acid-like substances from crude DNA extracts. Because sediments containing a high content of humic substances were used for DNA extraction with successful PCR results, this technique could also be applied to environmental samples containing lower contents of humic-acid-like substances, such as water and sewage (experiments in progress). Since both methods, that for direct extraction of DNA and that for purification of crude DNA, are relatively fast, they can be implemented by investigators who study in situ gene occurrence. These methods will also be of great benefit to scientists tracking genetically engineered microorganisms released into natural environments.

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REFERENCES

1. **Abbott, M. A., B. J. Poiesz, B. C. Byrne, S. Kwok, J. J. Sninsky, and G. D. Ehrlich.** 1988. Enzymatic gene amplification: qualitative and quantitative methods for detecting proviral DNA amplified in vitro. J. Infect. Dis. **158:**1158–1169.
2. **American Public Health Association.** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
3. **Arnheim, N., T. White, and W. E. Rainey.** 1990. Application of PCR: organismal and population biology. BioScience **40:**174–182.
4. **Bej, A. K., J. L. DiCesare, L. Haff, and R. M. Atlas.** 1991. Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. Appl. Environ. Microbiol. **57:**1013–1017.
5. **Bej, A. K., M. H. Mahbubani, J. L. DiCesare, and R. M. Atlas.** 1991. Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. Appl. Environ. Microbiol. **57:**3529–3534.
6. **Bej, A. K., R. J. Steffan, J. L. DiCesare, L. Haff, and R. M. Atlas.** 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl. Environ. Microbiol. **56:**307–314.
7. **De Leon, R., C. Shieh, R. S. Baric, and M. D. Sobsey.** 1991. Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction, p. 833–853. Proc. Water Quality Tech. Conf., San Diego, Calif., 1990.
8. **Hagelberg, E., and B. Sykes.** 1990. Ancient bone DNA amplified. Nature (London) **342:**485.
9. **Higuchi, R., C. H. von Beroldingen, G. F. Sensabaugh, and H. A. Erlich.** 1988. DNA typing from single hairs. Nature (London) **332:**543–546.
10. **Innis, M. A., K. B. Myambo, D. H. Gelfand, and M. A. D. Brow.** 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proc. Natl. Acad. Sci. USA **85:**9436–9440.
11. **Loh, E. Y., J. F. Elliott, S. Cwirla, L. L. Lanier, and M. M. Davis.** 1989. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. Science **243:**217–220.
12. **Mahbubani, M. H., A. K. Bej, R. Miller, L. Haff, J. DiCesare, and R. M. Atlas.** 1990. Detection of *Legionella* with polymerase chain reaction and gene probe methods. Mol. Cell. Probes **4:**175–187.
13. **Mahbubani, M. H., A. K. Bej, M. Perlin, F. W. Schaefer III, W. Jakubowski, and R. M. Atlas.** 1991. Detection of *Giardia* cysts by using the polymerase chain reaction and distinguishing live from dead cysts. Appl. Environ. Microbiol. **57:**3456–3461.
14. **Mead, D. A., N. K. Pey, C. Herrnstadt, R. A. Marcil, and L. M. Smith.** 1991. A universal method for the direct cloning of PCR amplified nucleic acid. Bio/Technology **9:**657–661.
15. **Pollard, D. R., W. M. Johnson, H. Lior, S. Tyler, and K. R. Rozee.** 1990. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. J. Clin. Microbiol. **28:**540–545.
16. **Rotbart, H. A.** 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. **28:**438–442.
17. **Sobsey, M. D., and A. R. Hickey.** 1985. Effects of humic and fulvic acids on poliovirus concentration from water by microporous filtration. Appl. Environ. Microbiol. **49:**259–264.
18. **Thurman, E. M., G. R. Aiken, M. Ewald, W. R. Fischer, U. Forstner, A. H. Hack, R. F. C. Mantoura, J. W. Parsons, R. Pocklington, F. J. Stevenson, R. S. Swift, and B. Szpakowska.** 1988. Isolation of soil and aquatic humic substances, p. 31–43. *In* F. H. Frimmel and R. F. Christman (ed.), Humic substances and their role in the environment. John Wiley and Sons, Ltd., New York.
19. **Tsai, Y.-L., and B. H. Olson.** 1990. Effects of Hg^{2+}, CH_3-Hg^+, and temperature on the expression of mercury resistance genes in environmental bacteria. Appl. Environ. Microbiol. **56:**3266–3272.
20. **Tsai, Y.-L., and B. H. Olson.** 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. **57:**1070–1074.
21. **Tsai, Y.-L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58:**754–757.
22. **Versalovic, J., T. Koeuth, E. R. B. McCabe, and J. R. Lupski.** 1991. Use of the polymerase chain reaction for physical mapping of *Escherichia coli* genes. J. Bacteriol. **173:**5253–5255.
23. **Webb, L., M. Carl, D. C. Malloy, G. A. Dasch, and A. F. Azad.** 1990. Detection of murine typhus infection in fleas by using the polymerase chain reaction. J. Clin. Microbiol. **28:**530–534.